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THE UNIVERSITY OF ALBERTA

POLLEN AND EMBRYO COMPETITION IN DOUGLAS-FIR

by

NOLA M. DAINTITH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

FOREST SCIENCE

EDMONTON, ALBERTA

SPRING 1987

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Abstract

Simple polyembryony is characteristic of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco). A number of embryos (one to four) are possible within any one ovule and these embryos may be genetically different. Only rarely does more than one embryo per ovule mature and this successful embryo is determined through competition, and not through random events. This conclusion is based on the results of a number of controlled pollinations using fresh pollen mixes composed of genetically different and identifiable pollen genotypes present in specific proportions. Although all pollen genotypes were equally represented in the fresh mixes (in terms of numbers), some pollen genotypes produced more of the successful embryos than others, indicating competitive advantages among pollen types. Stored pollen mixes were also used for controlled pollinations, and the results indicate that pollen vigor is an important factor in determining the most effective pollen parent. The pollen with the highest viability (as determined by respiration rate) produced more successful embryos than the other stored pollen types. Seed set of the fresh pollen mixes was significantly higher than seed set of the stored pollen mixes. These results have implications for supplemental mass pollination in Douglas-fir seed orchards.

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I. INTRODUCTION

Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) is native to North America, where two forms of the species are generally recognized. The coastal form (P. menziesii var. menziesii (Mirb.) Franco) occurs on the islands and mainland of the Pacific coast, while the interior form (P. menziesii var. glauca (Beissn.) Franco) is distributed throughout the Rocky Mountains from southwestern Alberta and central British Columbia, south through the western United States and into Mexico (Hosie, 1975).

The extensive distribution of Douglas-fir and its desirability as a lumber species make it commercially important in Oregon, Washington, California, Montana, Idaho and British Columbia. The demand for forest resources is expected to increase over the next 50 years, and these demands will inevitably be met by harvesting second growth forests (National Council on Gene Resources, 1982). This situation has created a great demand for genetically superior seed for reforestation purposes. Much of this seed will be produced in seed orchards, but there are problems of annually variable seed yields in terms of quantity and genetic quality. A better understanding of the reproductive biology of Douglas-fir may provide orchard managers with techniques to ensure a continuous and abundant supply of genetically superior seed.

The reproductive anatomy and reproductive development of Douglas-fir have been extensively described by Allen and

Owens (1972) and Owens (1973). The seed cone of Douglas-fir is a compound strobilus consisting of an axis bearing a series of spirally arranged bracts. A single, flattened ovuliferous scale is attached to the cone axis in the axil of each bract. Two ovules, which mature to form seed when successfully fertilized, are attached to the adaxial surface of the ovuliferous scale (Allen and Owens, 1972).

In Douglas-fir each ovule consists of an integument (which will develop into the seed coat) and a many-celled haploid female gametophyte (megagametophyte). At the time of fertilization, the female gametophyte is fully developed and contains stored nutrition and four to seven archegonia (Owens, 1973). A single egg cell develops within each archegonium; thus there is the potential for multiple fertilizations and multiple embryos within each ovule. The presence in each female gametophyte of more than one egg, and the potential for each egg, if fertilized, to produce an embryo is termed simple polyembryony (Willson and Burley, 1983).

Simple polyembryony is characteristic of almost all coniferous genera (Singh, 1978). The occurrence of polyembryony in gymnosperms is considered to be a form of developmental selection (Buchholz, 1926), for if each egg cell within an ovule were to be fertilized by male gametes from pollen grains of differing genotypes, several genetically different, viable embryos would be possible. Since only very rarely does more than one embryo mature in

one seed, there is competition among embryos. However, it is not only competition between the different embryos that determines the survivor; the successful embryo plus the pollen that produced it must be superior to the sum of the pollen and resultant neighboring embryos within the same ovule (Buchholz, 1922; Buchholz, 1926).

Competition is thought to be important in determining the embryo that attains the terminal position within the megagametophyte, and it is this embryo that is considered to be dominant in each ovule (Buchholz, 1926; Orr-Ewing, 1957). However, very little is actually known about competition and selection within the ovule of Douglas-fir, and it may be possible that an embryo may attain the dominant, terminal position as a result of chance events. In this study, I tested the null hypothesis: in Douglas-fir there are no competitive advantages between genetically different pollen parents in their ability to produce the embryo that will mature in the seed.

Gel electrophoresis is an invaluable technique for use in this type of study. Pollen from parents with unique enzyme markers can be used for controlled pollinations of selected maternal clones. The resultant embryos and their corresponding megagametophytes can then be electrophoretically assayed for the enzyme systems where these specific enzyme markers occur. In gymnosperms, the genetic maternal contribution to the embryo is identical to the genotype of the haploid megagametophyte, so by comparing

the electrophoretic phenotype of the megagametophyte to that of the embryo, it is possible to infer the genetic paternal contribution. Each pollen parent, being unique, can be readily identified based on its contribution to the embryo at its marker enzyme loci.

In this study, selected mother trees were control pollinated with pollen mixes. These mixes were comprised of known and unique pollen genotypes present in proportions specified by the mix ratio. Upon pollination with the mix, each ovule had available to it simultaneously pollen grains from each genotype in numbers proportional to the mix ratio. Therefore, proportional numbers of pollen grains of each pollen genotype should be taken into the micropylar canal. Assuming that this happens, there is the potential for the formation of a number of genetically different embryos within each ovule. If the genotype of the mature seed, as determined by gel electrophoresis, indicates that one pollen genotype produces the majority of successful embryos, then competition can be considered important and this pollen genotype is assumed to be highly competitive for whatever reason. However, if all of the pollen genotypes are responsible for producing the successful embryos and if the ratio of successful embryos produced by the different genotypes is similar to the ratio of the pollen genotypes in the mix, then the successful embryo is determined through random events.

A secondary part of this study assessed developmental events within the ovule through pollination, fertilization and early embryo development. The objective of this portion of the study was to determine if multiple embryos are produced within the ovule of Douglas-fir.

II. METHODS

Pollen Mix Formulations

Six Douglas-fir clones located in the MacMillan Bloedel clone banks on Vancouver Island were selected as pollen parents because of their unique and identifiable genotypes at four enzyme loci. These clones had been genotyped at a number of loci as part of a seed orchard contamination study (Fast *et al.*, 1986). The four loci (Malate dehydrogenase-3 (MDH-3), Malate dehydrogenase-2 (MDH-2), Glucose-6-phosphate dehydrogenase (G6P), and Phosphoglucomutase-2 (PGM-2)) were chosen because they are consistently well resolved, polymorphic loci in Douglas-fir. The pollen parents were homozygous at each of the four loci (with one exception), so the genotype of the male gamete produced by each pollen parent was known (Table 1).

For the six clones that were selected, stored pollen was available from MacMillan Bloedel for three clones and fresh pollen was available from four clones. Each pollen lot was assigned a number from one to six. The three stored pollen lots were designated as pollen types 1, 2, and 6, and the fresh pollen lots were designated as pollen types 1, 3, 4, and 5 (Table 1).

The stored pollen was collected in 1983 and stored in evacuated plastic pouches under conditions of low temperature (4 °C) and low humidity (R. Bower, pers. comm.). Due to the natural aging of pollen and the potential for

Table 1. Genotype of each pollen parent at the four selected enzyme loci.

Pollen Type	Clone bank-Clone no.*	Pollen Condition	Enzyme Loci G6P	MDH-3	MDH-2	PGM-2
1	6-321	Fresh/Stored	11	22	11	22
2	3-360	Stored	11	22	22	-
3	6-127	Fresh	11	33	11	-
4	2-053	Fresh	22	22	11	-
5	2-164	Fresh	11	22	11	13
6	2-123	Stored	22	22	11	-

* clone identification numbers used by MacMillan Bloedel.

inconsistencies in processing and storage, the germination capacity of pollen that has been stored for one or more years is generally variable (Goddard and Matthews, 1981). The rate of pollen respiration (Binder and Ballantyne, 1975) was used to estimate the vigor of each stored pollen type. Pollen vigor correlates with viability, so respiration rate measurements also provided an estimate of viability. A correlation coefficient of 0.77 between seed yield (filled seeds per cone) and respiration rate has been reported in Douglas-fir (Webber, 1987). There are problems, however, using respiration rate as a viability assay. Pollen lots may have comparable respiration rates and yet one pollen lot may have a lower proportion of live pollen grains compared to the others; the lack of live pollen grains being compensated for by the higher vigor of the live pollen. In order to obtain a better indication of viability, each stored pollen type was used singly to pollinate each mother tree, and the resultant seed yield was determined.

Six pollen mixes were used for the study. Four mixes were made with stored pollen and two with fresh pollen (Table 2). Stored and fresh pollens were not used in the same mixes due to potentially large differences in viability. Although there were differences in viability among the stored pollen types (Table 7, Results) it was necessary to use all three types in the mixes. Collection and storage time and conditions were similar for the fresh pollen, and the time between pollen collection and

Table 2. Pollen mix constituents and ratios of the constituents in the mix according to pollen weight.

Pollen Condition	Mix	Pollen Types	Mix Ratio
Stored	A	1,2,6	1:1:1
	B	2,6	1:1
	C	2,6	1:3
	D	2,6	3:1
Fresh	E	1,4,5	1:1:1
	F	1,3,4,5	1:1:1:1

pollination was very short, so the viability of all four pollen types should have been comparable.

Mixes A and B were made up of three and two of the stored pollen types, respectively. Mix A combined the three types in a 1:1:1 ratio based on pollen weight, and Mix B combined two pollen types in a 1:1 ratio based on pollen weight (Table 2). Combining Douglas-fir pollens by equal weight will result in approximately equal numbers of pollen grains of each type in the mix (Adams, 1982). Mixes C and D combined two stored pollen types in either a 1:3 or a 3:1 ratio based on pollen weight. The two fresh mixes, E and F, combined three and four of the fresh pollen types in 1:1:1 and 1:1:1:1 ratios, respectively. The pollen was weighed to the nearest 0.001 g and the constituent types were thoroughly mixed.

A check was performed to determine whether this method of formulating mixes based on pollen weight would result in there being proportionate numbers of pollen grains from each pollen type on the stigmatic tips after pollination. Each stored pollen type was stained a different color by soaking in solutions of either crystal violet, safranin O, or fast green for 30 to 60 seconds (Owens and Simpson, 1982). The pollen was filtered out of the solution and allowed to air dry. A sample of each color of pollen was viewed under the microscope to determine if staining had caused the pollen grains to clump together. Small clumps were infrequent and were broken down as necessary. Pollen of each color was

carefully weighed to the nearest 0.001 g and thoroughly combined to make a 1:1:1 ratio mix. This mix was used to pollinate receptive seed cones on two branches that had been isolated in pollination bags on a single Douglas-fir tree at the University of Victoria in Victoria, B.C.. The same technique and amount of pollen were used to pollinate these enclosed seed cones and those on the study trees. Following pollination, five cones were sampled from the two bags. Each cone was carefully dissected so as not to disturb the pollen grains on the stigmatic tips. Thirty stigmatic tips were sampled from all sides of the central portion of each cone. Each tip was viewed under a dissecting microscope, and the number of pollen grains of each color adhering to the tip was recorded.

The expected ratio of colored pollen grains on the stigmatic tips was 1:1:1. A log-likelihood ratio (G) statistic for goodness of fit (Sokal and Rohlf, 1981) was calculated from the collected data to determine if there was any significant departure from this expected ratio.

Controlled Pollination Methods

Eight Douglas-fir trees located in the B.C. Ministry of Forests and Lands' Dewdney Seed Orchard were selected as maternal parents. The Dewdney Orchard is a first generation Douglas-fir orchard containing material collected from high elevations (670-1070 m). It is located on the Saanich Peninsula 15 km north of Victoria, B.C. (Ministry of

Forests, 1980). Seed produced in this orchard is utilized in the coast/interior transition zone of the province. The selected trees were approximately 18 years old and were four to six meters tall. They were selected based on an abundance of seed-cone buds and a history of late receptivity.

Eighteen branches, each bearing five to eight seed-cone buds, were selected in the lower portion of the crown of each mother. Pollen-cone buds were removed from the selected branches prior to isolation. Each branch was isolated within a windowed kraft pollination bag when the seed-cone buds had become noticeably swollen but while they were still enclosed within bud scales. Branches were bagged over a three-day period from April 12 to April 15, 1985, due to phenological differences among the mother trees. Small pieces of insecticide strip (Vapona no-pest strip) were placed in each pollination bag to control any insects that were on the branches at the time of bagging.

Eleven treatments were applied to the eighteen selected branches (Table 3). Each mother was control pollinated with the six pollen mixes (each replicated two times) and each of the three stored pollen types for a total of nine treatments. The tenth treatment was a control and received no pollen. The two remaining branches were reserved for the developmental study and were pollinated with an additional lot of stored pollen obtained from MacMillan Bloedel. The viability of this pollen was similar to the viability of the pollen types used in the stored mixes (Table 7, Results).

Table 3. Pollination treatments applied to eighteen selected branches on each mother tree.

Branch Number	Treatment	Replication
1	Mix A	1
2	Mix B	1
3	Mix C	1
4	Mix D	1
5	Mix A	2
6	Mix B	2
7	Mix C	2
8	Mix D	2
9	Control	
10	Stored Pollen 1	
11	Stored Pollen 6	
12	Stored Pollen 2	
13	Developmental Study	
14	Developmental Study	
15	Mix E	1
16	Mix F	1
17	Mix F	2
18	Mix E	2

The same method of pollination was used to treat all branches. The developmental study was done to determine the potential for, and the actual occurrence of, multiple embryos within a single ovule.

The phenology of each branch was monitored daily and the date of bud burst, B+0 (when the conelets had emerged one-half to two-thirds of the way out of their scales), of the majority of the enclosed seed-cone buds was recorded (Owens *et al.*, 1981). Each branch was randomly assigned a colored marker that corresponded to a specific treatment. All branches, except those which were pollinated with the fresh mixes, were pollinated four days after B+0, the optimal time of pollination being a four-day period from B+0 to B+4 (Owens *et al.*, 1981). Most trees were pollinated over a period of two days due to differences in phenology among branches; pollinations were made from April 18 to April 22, 1985. Fresh pollen mixes were not available until April 23, 1985, so branches designated for these treatments were five to eight days past B+0 when they were pollinated, and the receptivity of the conelets was likely less than optimal. Trees 1 and 5 were not pollinated with the fresh mixes, since they were nine days beyond bud burst (B+0).

To ensure that an abundance of pollen was available to the enclosed cones, 0.2 g of pollen was used per pollination bag (Owens *et. al.*, 1981). A small container was devised for use in the orchard to measure a volume of pollen with a weight of approximately 0.2 g.

A hypodermic syringe fitted with a rubber squeeze bulb was used to apply pollen to the isolated branches. Air was forced into the syringe through a hooked glass tube that acted to create a cloud of pollen within the syringe so that all of the pollen was not expelled with one squeeze. The needle was inserted through the pollination bag and directed at the receptive conelets. The bulb was squeezed several times to ensure each conelet received some pollen. When the syringe was empty, the bulb was squeezed several more times to further distribute the pollen that had settled in the bag. Needle holes in the pollination bags were sealed with tape to prevent contamination by outside pollen. Approximately five weeks after pollination the pollination bags were replaced with mesh insect bags.

Developmental Study

Cones were collected from the developmental study branches over two periods in June. The first collection period was approximately six weeks after pollination at approximately the time of fertilization (Owens, 1973), and the second collection period was approximately two weeks later. The cones were, depending on the collection period, subsampled for either the number of archegonia, elongated pollen grains, or embryos within each ovule. Since all of the treatment methods were similar, the results from these cones could then be extrapolated to the study cones.

Two cones from each of the two developmental study branches were collected from each mother tree from June 13 to 20, 1985. At this time the megagametophytes were fully developed. Twenty ovules were sampled from all sides of the central portion of each cone. Ovules in the basal and distal portions of the cone were not sampled as these ovules are often sterile (Ho, 1980). The integument tip of each selected ovule was carefully bisected under a dissecting microscope to open up the micropylar canal. The number of elongated pollen grains in the micropylar canal or attached to the nucellus was counted, along with the number of pollen grains that had already discharged their contents into the archegonia.

Eight more ovules were sampled from each of the four cones from each tree. These ovules, along with eight ovules sampled from each of two open-pollinated cones from each tree were dissected to determine the number of archegonia present in each ovule. A thin freehand section was taken from the tip of each female gametophyte so that the cross-section of each archegonium was visible in the section. Staining of these sections was not necessary as the archegonia were clearly visible when viewed through a dissecting microscope.

Three more cones were collected from the developmental bags on each tree on June 28 and 29, 1985. Ten ovules showing evidence of embryo development were sampled from the central portion of each cone. Some of the cones sampled

showed a high incidence of ovule abortion, and it was difficult to find ten suitable ovules in these cones. Using a dissecting microscope and a new, thin razor blade, a longitudinal freehand section was taken from each ovule. The sections were made to partially open both sides of the corrosion cavity, and care was taken not to dislodge the developing embryos within the cavity. These sections were fixed in Carnoy's solution (Johansen, 1940) for at least one hour and then cleared in boiling lactic acid for approximately 35 minutes. The sections were stained for 15 minutes in Delafield's hematoxylin, a stain which stains meristematic tissue (Johansen, 1940). The sections were viewed under a light microscope, and the number of developing embryos was recorded.

Cone Collection and Seed Yield

Mature cones were collected on August 20 and 21, 1985, just prior to seed shed. The cones were placed in open, labelled paper bags and allowed to dry at room temperature. When the cones began to open, the seeds were extracted by hand and kept separate by cone.

The seed yield (number of filled seeds per cone (fspc)) produced by each stored pollen type and by the control cones was determined by longitudinally cutting the "full" seeds from each cone. Only those seeds that contained a firm, white, healthy megagametophyte and a healthy embryo were counted. An analysis of variance was performed on the fspc

data obtained from each treatment to determine if there were significant differences among the stored pollen types in their ability to set seed on each mother tree.

The number of "full" seed extracted from cones from each of the pollen mixes was recorded. This number, and the number of sound seed that underwent electrophoresis for each treatment and tree, were used to estimate the percent seed set for each treatment. Orthogonal comparisons (Steel and Torrie, 1980) were made to determine if any significant differences existed in these seed set data.

Electrophoretic Methods

Seed extracted from the cones pollinated with the pollen mixes was hydrated for approximately one week. Megagametophytes and embryos from each seed were homogenized separately in extraction buffer (Yeh and Layton, 1979) and electrophoretically assayed for one to three enzyme loci using starch gels (12.5% Connaught Hydrolyzed Starch) and ordinary staining procedures (Table 4). The enzyme systems were dependent upon the pollen mix and were those at which the pollen genotypes in the mix were identifiable (Table 5). For example, mixes B, C, and D each contained pollen types 2 and 6, whose genotypes were homozygous and identifiably different at the G6P locus (Table 5). Thus it was possible to identify the pollen parent of each embryo resulting from pollination with each mix at the loci indicated.

Table 4. Enzyme systems assayed and corresponding buffer systems and staining procedures used in electrophoresis.

Enzyme	E.C. Number	Buffer System'	Stain Reference
PGM-2	2.7.5.1.	A	Yeh and O'Malley (1980)
MDH-2	1.1.1.37.	B	Yeh and O'Malley (1980)
MDH-3	1.1.1.37.	B	Yeh and O'Malley (1980)
G6P	1.1.1.49.	C	Yeh and O'Malley (1980)

'Buffer systems:

A Electrode buffer: sodium borate pH 8.1
Gel buffer: tris-citrate pH 8.65; Power 300 volts
(Schaal and Anderson, 1974)

B Electrode buffer: tris-citrate pH 7.0
Gel buffer: tris-citrate pH 7.0; Power 200 volts
(Siciliano and Shaw, 1976)

C Electrode buffer: tris-citrate pH 7.0
Gel buffer: L-histidine-HCl pH 7.0; Power 200 volts
(Namkoong *et al.*, 1979)

Table 5. Enzyme systems used to identify the effective pollen genotypes in each mix.

Mix	Enzyme Systems
A	G6P, MDH-2
B	G6P
C	G6P
D	G6P
E	G6P, PGM-2
F	G6P, PGM-2, MDH-3

The number of embryos produced by each pollen type was recorded for each mother tree and treatment. Assuming the null hypothesis of no competition between pollen genotypes, the expected ratio of embryos is the ratio of pollen types in the pollen mix (i.e., the mix ratio). For example, the expected ratio of embryos produced by the three pollen types in mix E is 1:1:1. For each mix, log-likelihood ratio (G) statistics for goodness of fit were calculated to determine if there were any significant departures from the expected ratios. The G-statistic was chosen over the Chi-square statistic because of its additivity. The results of several G-tests can be summed to yield meaningful results (Sokal and Rohlf, 1981), so that in this study, the G-statistics can be summarized over the mother trees to yield a total G for each treatment. Also, total G-statistics can be partitioned into separate components in a manner analogous to that practiced in analyses of variance so that factors contributing to heterogeneity in the data can be explained.

III. RESULTS

Developmental Study

Results obtained from the dissection of ovules to expose a cross-section of the megagametophyte revealed that the number of archegonia within each ovule ranged from two to seven. Of the 420 ovules sampled from the eight mother trees, 196 or 46.7 percent contained four archegonia (Table 6). Approximately 33 percent of the ovules contained five archegonia, nine percent contained six archegonia and only one ovule was found to contain seven archegonia. These results are comparable to those of Owens (1973), who reported four to seven archegonia in Douglas-fir. However, in this study some ovules were found to contain only two or three archegonia (approximately one percent and ten percent, respectively).

The average number of elongated pollen grains found within the micropylar canal (at approximately the time of fertilization) was 1.39, with a range of one to three (Table 6). These numbers include those pollen grains that had already expelled their contents into an archegonium and were shrivelled on the tip of the nucellus. Pollen grains that had not yet elongated were not recorded.

Two hundred and four ovules were dissected to expose the corrosion cavity and the developing embryos within. The average number of embryos per ovule was 1.78, with a range of one to four (Table 6). Approximately 33 percent of the

Table 6. Number of archegonia, elongated pollen grains and embryos within the sampled ovules.

	Number in Each Ovule	Number of Ovules	Percent of Ovules	Average per Ovule	s.e.
Archegonia	2	3	0.8		
	3	43	10.2		
	4	196	46.7	4.40	0.040
	5	140	33.3		
	6	37	8.8		
	7	1	0.2		
Elongated Pollen Grains	1	286	63.8		
	2	149	33.2	1.39	0.026
	3	13	3.0		
Embryos	1	67	32.8		
	2	115	56.4	1.78	0.045
	3	21	10.3		
	4	1	0.5		

ovules contained one embryo, 56 percent contained two embryos, ten percent contained three embryos and less than one percent contained four embryos. Cursory observations made while dissecting embryos from megagametophytes for electrophoresis support these results of the occurrence of multiple embryos. Approximately ten percent of the ovules from mature cones contained two or more embryos. In some ovules the embryos appeared to be equally developed, while in others one embryo was obviously dominant. In cases where two large embryos were present in the mature seed, the electrophoretic results often revealed that each embryo had been fertilized by a different pollen type in the mix.

Genotype Competition Study

Following controlled pollination with the colored pollen mix, 350 red pollen grains, 361 green pollen grains and 399 blue pollen grains were counted on the 150 stigmatic tips sampled from five cones. The overall G-statistic was 11.63, which is insignificant ($p<0.05$) when compared to the appropriate Chi-square value of 18.31. Therefore, the mix formulation method and the pollination method was assumed to be sound as there was no significant departure from the expected 1:1:1 ratio of colored pollen grains on the stigmatic tips. Sixty-three percent of the stigmatic tips contained at least one pollen grain of each pollen type (as indicated by pollen color), and 90 percent of the stigmatic tips contained pollen grains of two pollen types.

The respiration rate of each stored pollen type was determined just prior to controlled pollination (Table 7). Only one measurement was made for each pollen type as pollen supply was limited and, therefore, the significance of differences in the rates could not be determined. Pollen 6 had a higher respiration rate, being approximately three times higher (5.72 $\mu\ell O_2$/min/g), than the rates of either pollen 1 or pollen 2 (2.14 and 1.95 $\mu\ell O_2$/min/g, respectively).

Pollen 6, however, did not produce the highest seed yield when it was used singly to pollinate each mother tree. The seed yield, expressed as mean filled seed per cone (fspc), for pollen 6 was 12.5 averaged over the eight trees (Table 8). The average seed yields for pollen 1 and pollen 2 were 13.6 and 7.8, respectively.

Unequal numbers of cones per treatment were used to calculate the mean fspc values. In order to do an analysis of variance, it was necessary to randomly delete cones until the treatment sample sizes (number of cones) were equal for each tree. Sample sizes were allowed to vary between trees. Significant differences ($p<0.05$) were found in the mean fspc data between pollen types, between mother trees, and between pollen types and trees.

No one pollen type consistently produced the highest seed yield on all of the mother trees, although pollen 1 was most productive on five trees (Table 8). The considerable tree-to-tree variation in the data made it difficult to

Table 7. Respiration rates of the stored pollen types.

	Rs ($\mu\ell O_2$/min/g)
Pollen 1	2.14
Pollen 2	1.95
Pollen 6	5.72
Pollen for developmental study	6.08

Table 8. Mean number of filled seeds per cone (fspc) produced by each stored pollen type on each mother tree.

Tree Number	Pollen 1	Pollen 2	Pollen 6
1	15.1	1.3	12.9
2	20.2	1.4	17.4
3	4.4	1.6	19.4
4	7.5	5.2	2.8
5	6.1	5.9	7.7
6	23.8	23.0	16.4
7	13.5	18.8	7.4
8	18.2	5.4	16.0
Average	13.6	7.8	12.5

determine any trends. For example, the seed yield produced by each of the three pollen types was similar on tree 5, whereas on tree 3 the seed yields produced by the pollen types were quite variable. If the seed yield results had not been so variable, they could have been used to adjust the stored pollen mix data to account for differences in viability among the constituent pollen types.

The embryos and megagametophytes from more than 6250 seeds were assayed using gel electrophoresis. Overall, less than two percent of the seed that was electrophoretically assayed produced results that were too faint or too ambiguous to score. Using the electrophoretic results, each resultant embryo was classified according to the pollen type that produced it (Tables 9 and 10). For example, 135 mature embryos were produced by tree 1 upon pollination with Mix A, and electrophoretically assayed for two enzyme loci. Of this total number of embryos from the two replications, 30 embryos were produced by pollen 1, 17 embryos were produced by pollen 2, and 88 embryos were produced by pollen 6. The ratio of embryos produced by the three pollen types was 1.8:1:5.2 (Table 9). Only those trees that produced samples of adequate size (at least 30 seed for the two replications) were reported (Tables 9 and 10) and used in the statistical analyses (Table 11).

Pollen 6 produced a majority of the resultant embryos upon pollination with stored mixes A, B and C (Table 9). In Mix D, the mix ratio was 3:1 against pollen 6 and pollen 6

Table 9. Number and ratio of embryos produced by each stored pollen mix on each tree as determined by electrophoresis of mature seed contents.

Mix	Mix Ratio	Pollen Types	Tree	No. of Embryos From Each Pollen Type	Ratio of Embryos
A	1:1:1	1,2,6	1	30,17,88	1.8:1:5.2*
			2	77,36,79	2.1:1:2.2*
			3	73,28,91	2.6:1:3.2*
			5	14,11,50	1.3:1:4.5*
			6	87,46,103	1.9:1:2.2*
			8	36,14,26	2.6:1:1.9*
B	1:1	2,6	1	5,48	1:9.6*
			2	30,118	1:3.9*
			3	40,167	1:4.2*
			6	28,89	1:3.2*
			7	27,76	1:2.8*
			8	78,139	1:1.8*
C	1:3	2,6	1	12,106	1:8.8*
			2	10,49	1:4.9
			3	25,216	1:8.6*
			5	16,121	1:7.6*
			6	25,117	1:4.7*
			7	22,66	1:3
			8	11,76	1:6.9*
D	3:1	2,6	2	116,75	1.5:1*
			3	57,124	1:2.2*
			4	43,40	1.1:1*
			5	23,75	1:3.3*
			6	153,98	1.6:1*
			7	89,61	1.5:1*

* significant ($p<0.05$) departure from expected mix ratio.

Table 10. Number and ratio of embryos produced by each fresh pollen mix on each tree as determined by electrophoresis of mature seed contents.

Mix	Mix Ratio	Pollen Types	Tree	No. of Embryos From Each Pollen Type	Ratio of Embryos
E	1:1:1	1,4,5	2	56,41,53	1.4:1:1.3
			3	62,50,56	1.2:1:1.1
			4	83,70,77	1.2:1:1.1
			6	80,46,62	1.7:1:1.3*
			8	134,106,122	1.3:1:1.2
F	1:1:1:1	1,3,4,5	2	94,90,51,89	1.8:1.8:1:1.7*
			3	72,23,38,45	3.1:1:1.7:2.0*
			4	79,91,57,78	1.4:1.6:1:1.4*
			6	87,79,64,76	1.4:1.2:1:1.2
			7	16,14,10,10	1.6:1.4:1:1
			8	105,101,73,132	1.4:1.4:1:1.8*
F adj	1:1:1	1,3,5	2	94,90,89	1.1:1:1
			3	72,23,45	3.1:1:2.0*
			4	79,91,78	1:1.2:1
			6	87,79,76	1.1:1:1
			7	16,14,10	1.6:1.4:1
			8	105,101,132	1:1:1.3

* significant ($p<0.05$) departure from expected mix ratio.

still produced more embryos than pollen 2 on trees 3 and 5. On the other four trees, pollen 6 produced fewer embryos than pollen 2 although the differences were not great. These results were not surprising considering the respiration rate measurements of the three stored pollen types (Table 7); the ratio of respiration rates for the three types being 1.1:1:2.9.

It is apparent from these results that the pollen type with the highest respiration rate (pollen 6) was the most effective parent in the stored pollen mixes, and the pollen with the lowest respiration rate (pollen 2) was the least effective. While there was considerable variation among trees in the embryo ratios produced by a mix, the ratios generally deviated in the same direction over all trees, with the exception of the Mix D ratios. For example, the embryo ratios for Mix B ranged from 1:1.8 on tree 8 to 1:9.6 on tree 1, with pollen 6 producing the most embryos (Table 9). This would indicate that some male-female crosses produce embryos that are likely to be more successful compared to embryos resulting from other crosses. The embryos produced by pollen 6 and mother tree 1 were very successful (producing approximately nine times more embryos than pollen 2) compared to the embryos produced by pollen 6 and mother tree 8.

As a result of the differences in viability, the observed embryo ratios for the stored mixes deviated considerably from the expected ratios. In comparison, the

ratios of embryos produced by the fresh mixes E and F did not deviate as drastically from the expected ratios (Table 10). For both fresh mixes, pollen 1 was the most successful pollen parent, while pollen 4 was the least successful parent.

Log-likelihood ratio (G) statistics were calculated using the embryo data from each treatment and tree and these individual G-statistics were summed over the mother trees to yield a total G (G_T) for each treatment (the significant individual tree G-statistics are identified in Tables 9 and 10). An interaction, or heterogeneity, G-test was also performed in which the total G was decomposed into two components, a G for heterogeneity (G_H), and a pooled G (G_P). The heterogeneity G tests whether or not the embryo ratios from all mother trees are homogeneous, while the pooled G tests the goodness of fit to the expected ratio of the data pooled from all trees (Table 11). Assuming the null hypothesis of no competition among pollen genotypes, the expected ratio is the mix ratio.

The total G, the pooled G, and the heterogeneity G were all highly significant ($p<0.05$) for the stored mixes A, B, C, and D, and for fresh Mix F. For the data as a whole from each of these mixes, the significant departures in the observed embryo ratios from the expected mix ratios were attributable both to the significant heterogeneity in the embryo ratios between mother trees (which was evident in Tables 9 and 10), and to the significant lack of fit of the

Table 11. G-statistics computed for each treatment (mix).

Mix	G_H	G_P	G_T
A	41.68*	144.97*	186.65*
B	26.85*	228.28*	255.13*
C	14.48*	65.25*	79.73*
D	80.78*	265.73*	346.51*
E	3.19	14.40*	17.59
F	35.59*	40.10*	75.69*
F adj.	31.60*	3.59	35.19*

* denotes significance at $p<0.05$

pooled data to the expected ratios. For these mixes, a majority (67 percent to 100 percent) of the individual G-statistics calculated from the individual tree results were significant (Tables 9 and 10).

The heterogeneity G for Mix E and all but one of the five individual tree G-statistics were found to be insignificant, while the pooled G was significant. The insignificant heterogeneity G indicates that the deviations in the embryo ratios from the expected ratios were all in the same direction, and not significantly different from each other, for all mother trees. Since the results from all mother trees deviated in the same direction, the pooled data, being based on a large sample size, show this deviation clearly and yield a significant pooled G. The total G remains insignificant, albeit high (17.59 compared to the appropriate Chi-square value of 18.31), in spite of the significant pooled G.

The raw data (Table 10) show that, with only one exception (Mix F, tree 3), pollen 4 was the least successful fresh pollen type in mixes E and F on all mother trees. To determine if it was only pollen 4 in Mix F that was responsible for the overall significant departure from the expected ratio, pollen 4 data were deleted from the Mix F data (Mix F adj. in Tables 10 and 11) and the G-statistics were recalculated. The heterogeneity G and the total G remained significant while the pooled G was surprisingly insignificant. This apparent anomaly occurred because the

deviations from the expected ratios were not all in the same direction for all trees and the individual ratios were heterogeneous in such a way that they tended to compensate for the deviations in each other. Thus the pooled data fit the expected ratio. Only one of the six individual G-statistics was significant for the adjusted data (Table 10), but the lack of fit of this individual ratio to the expected ratio was great enough (the individual G accounted for 76 percent of the total G) to cause the total G to be significant.

Orthogonal comparisons made on the percent seed set data for the mix treatments indicated that the only significant difference was between the percent seed set for the fresh mixes and the stored mixes. There were no significant differences among the fresh mixes, or among the stored mixes, in their ability to set seed. The average seed set for the stored mixes was 29.8 percent, compared to the average seed set of 52.3 percent for the fresh mixes.

IV. DISCUSSION

Results of the developmental study indicate that there is considerable potential for the formation of multiple embryos within any one ovule of Douglas-fir. Most ovules (80 percent) contain either four or five archegonia, and numerous pollen grains (up to a maximum of nine) can be taken into the micropylar canal (Owens *et al.*, 1981; Silen, 1978). The number of embryos that are actually formed is generally less than this potential due to a number of factors. An average of less than four pollen grains usually are taken into the micropyle (Owens *et al.*, 1981), and not all of the engulfed pollen grains will elongate. In this study, an average of 1.39 elongated pollen grains were found in the micropyle. Ho (1980) and Silen (1978) reported an average of 1.6 and 1.8 elongated pollen grains, respectively, within the micropyle. These numbers may be considered to be minimum numbers, since unelongated pollen grains in the micropyle at the time of sampling may have elongated at a later date and participated in fertilization.

In spite of this low average number of elongated pollen grains reported in the micropyle, a majority (67.2 percent) of the ovules sampled after fertilization contained more than one, and as many as four, embryos; the average number being 1.78. Orr-Ewing (1957) also reported the occurrence of multiple embryos within ovules of Douglas-fir, counting 235 and 231 embryos in 92 and 75 fertilized ovules, respectively. Whether the embryos within an ovule were

genetically different in this study is unknown. In a few cases, however, two embryos of sufficient size were present in the mature seed and the electrophoretic results revealed that each embryo had been fertilized by a different pollen type in the mix. Generally when more than one embryo was present in the mature seed, only one embryo was large enough to be electrophoretically assayed so it was difficult to determine the frequency of these genetically different "twins" or "triplets". Based on these results, genetically different embryos did occur within a single ovule, thus providing the genetic diversity for competition and selection.

Results obtained from controlled pollination with the colored pollen mix indicated that, upon pollination with the study mixes, pollen grains of each genotype would have been present in numbers directly proportional to the mix ratio on the stigmatic tips. Since all of the pollen grains were delivered to the cones within seconds, they should have been captured in the stigmatic hairs at approximately the same time; therefore, there would have been no "first-on, first-in" advantages between any of the pollen types. Pollen arriving at the stigmatic tips first has been reported to have an advantage over pollen arriving later in completing subsequent steps to fertilization (Webber and Yeh, 1987). Thus, pollen grains of each pollen type would have been equally likely to have been taken into the micropyle in proportions dictated by the mix ratio. Once in the

micropyle, whether or not a pollen grain elongated and fertilized an egg to produce a viable embryo would largely depend on its vigor.

Vigor was a very important factor in determining the success of the pollen types in the stored pollen mixes. Pollen 6 had the highest respiration rate and was consistently the most successful pollen type in the stored mixes. In only one instance did pollen 6 produce fewer embryos than expected based on the mix ratio. Pollen 2, in comparison, had the lowest respiration rate and consistently produced fewer embryos than expected.

While pollen 6 did not produce, on average, the highest seed yield when it was applied singly to the mother trees, the seed yield data were so inconsistent that it was difficult to relate the success of any pollen type on any tree to the success of that same pollen type in a mix on that tree. There are a number of possibilities that may explain the inconsistencies in the seed yield results. The use of a syringe as the pollinating apparatus made it difficult to ensure that the pollen was evenly distributed to all conelets and all of the stigmatic tips within each conelet, and that all stigmatic tips did, in fact, receive some pollen. Because of the natural variation in phenology among cones and even within cones, the development of stigmatic tips was not uniform within a pollination bag. Even though all pollinations were made four days after bud burst, the variation in stigmatic tip development at this

time among trees, branches and cones likely contributed to the variable seed yield results. Better physical control of the pollinations and, most important, replicated treatment with each stored pollen type on each mother tree may have resulted in less variable results.

The G-statistics for the stored pollen mixes were all highly significant, but the significant departures of the results from the expected mix ratios were predominantly due to the differences in viability among the stored pollen types. There were simply more viable pollen grains of pollen 6 in the mixes and therefore available for fertilization as evidenced by the number of successful embryos produced by pollen 6. Based on these results, no statement can be made concerning competitive advantages among the pollen genotypes in the stored pollen mixes. However, it is apparent that pollen viability, as determined by respiration rate, does give a good indication of how effective (in terms of producing successful embryos) a pollen genotype will be in a mix.

All of the fresh pollen types were assumed to be of comparable viability because of the consistency in pollen collection and handling, and the very short time between collection and pollination. For the Mix E data as a whole, the ratio of embryos produced did not depart significantly from the expected mix ratio. Considering the insignificant total G, the null hypothesis cannot be rejected. However, the individual ratios from the mother trees all deviated in

the same direction causing a significant deviation in the pooled data. This consistent deviation in the ratios resulted because pollen 4 was the least effective pollen type, and pollen 1 was the most successful pollen type, over all trees. While this would suggest that competition was occurring among the pollen genotypes, overall the deviations were not sufficient to cause the total G-statistic to be significant, although it was high.

All of the G-statistics computed from the Mix F data were significant, indicating a significant departure from the hypothesized 1:1:1:1 ratio, and significant heterogeneity in the data between mother trees. Therefore the null hypothesis was rejected and it was apparent that there were competitive advantages between the pollen genotypes in the mix. As in Mix E, pollen 4 consistently produced fewer of the successful embryos compared to the other pollen types. When the pollen 4 data were deleted, the total G was considerably reduced but it remained significant, mainly due to one highly significant individual G-statistic. While pollen 4 was not solely responsible, it did contribute to, the overall lack of fit to the expected ratio. The significant adjusted total G would indicate that there were competitive advantages between the remaining three pollen types. However, there was significant heterogeneity in the ratios between trees, and no one pollen type was the most effective parent over all trees, or the least effective parent as pollen 4 had been.

Considering these fresh pollen mix results, pollen 4 appears to be less "competitive" than the other three fresh pollen types. In Mix F, pollen 4 produced few enough of the successful embryos to cause a significant departure from the expected ratio. The ineffectiveness of pollen 4 in Mix E was not sufficient enough to cause a significant departure, overall, from the expected ratio. However, the deviations in the individual tree ratios were all uniform and in the same direction, and all of the ratios pooled showed a deficiency in the number of embryos produced by pollen 4. With the addition of one more pollen type in Mix F, there were more genotypes (although not any more embryos) competing for the sole right to develop and form the mature seed. When only two other pollen types presented competition in Mix E, the number of embryos produced by pollen 4 was less than, but statistically comparable to, the number of embryos produced by each of the other two pollen types. Competition was increased with the addition of one more pollen genotype in Mix F, and pollen 4 was not able to compete as well; therefore, there was a decline in the number of successful embryos produced by pollen 4 relative to the number of embryos produced by the other pollen types.

Based on the fresh pollen mix results, there are differences in competitive abilities among Douglas-fir pollen genotypes, with some pollen genotypes being more likely to produce the successful embryo than others. The differential competitive ability may be due to genetic

differences between pollen types, or to the male-female interaction in the embryos, with some crosses resulting in embryos that are more competitive than others. It is also possible that competitive ability may be the result of physiological differences (i.e., differential pollen vigor). Either way, it is evident that the mature embryo in a Douglas-fir seed is determined through competition and selection, and not through chance events. Whether competition and selection occurs only in the stages of early embryo development (post-zygotic), or whether competition begins with the elongation of pollen grains in the micropyle (pre-zygotic) and continues through embryo development could not be determined. Mulcahy (1987) speculates that much of the competition and selection in gymnosperms takes place at the embryo stage. As evidenced in this study, some pollen parents had varying success rates over the different mother trees. Some crosses appeared to produce embryos that were more successful than others, indicating that competition among embryos may be more important than pollen competition in determining the survivor.

The occurrence of competition among the genetically different embryos that are possible due to simple polyembryony can be beneficial to the species. In the event that early embryo abortion is high, the production of multiple embryos may protect against the production of a comparatively high proportion of empty seed. Simple polyembryony and embryo competition play an important role

in ameliorating the effects of self-pollination. Self pollen will germinate normally and participate in fertilization (Orr-Ewing, 1957), but because selfed embryos in Douglas-fir have low viability (Sorensen, 1982), they would be eliminated through competition and selection. In this way, the species allows for the inevitable occurrence of natural self-pollination by wind, but with a low production of selfed seedlings, which are generally less vigorous. Similarly, embryos homozygous for defective or lethal genes would also be eliminated.

Based on the results of this study, it is possible to make some recommendations for the use of supplemental mass pollination (SMP) in Douglas-fir seed orchards. Potential benefits from SMP are increases in seed yield (especially where orchard pollen cloud density is low), as well as increases in the genetic quality of the seed produced (Franklin, 1971; Denison and Franklin, 1975; Bridgwater and Trew, 1981; Bridgwater and Bramlett, 1982; Wheeler and Jech, 1986). If SMP is to successfully improve the genetic quality of orchard seed, every effort must be made to increase the probability of pollination by the selected parents that are supplementally applied. The supplemental pollen must, therefore, be of the highest possible quality if it is to be competitive with the fresh background pollen in the orchard. If it is possible, fresh pollen collected and processed just prior to pollination should be used. Seed set can be improved using fresh pollen compared to using stored pollen,

and fresh pollen will not be at a disadvantage (in terms of viability) competing with the background pollen.

If only stored pollen is available from the selected parents for SMP, as is often the case, pollen viability tests should be done prior to pollination to ensure that each pollen lot has similar viability and that, overall, viability is high. It may be necessary to adjust the pollen mix formulation to compensate for differences in viability, especially if the differences are large. If pollen from one of the selected parents has a considerably higher respiration rate and the mix is not adjusted to account for this, then it is conceivable that this pollen type could be responsible for a majority of fertilizations. This not only defeats the purpose of using a number of pollen parents in the SMP mix, it also reduces the genetic base of the seed that is produced. As Webber and Yeh (1987) stated, pollen that has been collected for storage and future pollinations must be maintained at the highest possible fertility.

Even if all pollens in the SMP mix, whether fresh or stored, are of equal viability, some genotypes will be more competitive than others and produce more of the successful embryos. The occurrence of multiple embryos by simple polyembryony provides the genetic diversity for competition and selection; therefore, it should be theoretically possible to increase selective pressures by using only the best parents as pollen sources for SMP (Franklin, 1971). Ensuring that adequate pollen is applied during SMP is

equally as important as the selection of pollen parents and the condition of the pollen. If pollen engulfed into the micropyle is limited and only one embryo is produced in a majority of the ovules, then the benefits of simple polyembryony and embryo competition will not be realized.

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